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# Membrane Anchoring of the Autoantigen GAD<sub>65</sub> to Microvesicles in Pancreatic $\beta$ -cells by Palmitoylation in the NH<sub>2</sub>-Terminal Domain

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**Abstract.** Pancreatic  $\beta$ -cells and  $\gamma$ -aminobutyric acid (GABA)-secreting neurons both express the enzyme glutamic acid decarboxylase (GAD) which is a major target of autoantibodies associated with  $\beta$ -cell destruction and impairment of GABA-ergic neurotransmitter pathways. The predominant form of GAD in pancreatic  $\beta$ -cells, GAD<sub>65</sub>, is synthesized as a soluble hydrophilic molecule, which is modified to become firmly membrane anchored. Here we show by immunogold electron microscopy that GAD<sub>65</sub> is localized to the membrane of small vesicles which are identical in size to small synaptic-like microvesicles in pancreatic  $\beta$ -cells.

The NH<sub>2</sub>-terminal domain of GAD<sub>65</sub> is the site of a two-step modification, the last of which results in a firm membrane anchoring that involves posttranslational hydroxylamine sensitive palmitoylation. GAD<sub>65</sub> can be released from the membrane by an apparent enzyme activity in islets, suggesting that the membrane anchoring step is reversible and potentially regulated. The hydrophobic modifications and consequent membrane anchoring of GAD<sub>65</sub> to microvesicles that store its product GABA may be of functional importance and, moreover, significant for its selective role as an autoantigen.

THE  $\gamma$ -aminobutyric acid (GABA)-synthesizing enzyme glutamic acid decarboxylase (GAD) is expressed at comparable levels in GABA-secreting neurons and in pancreatic  $\beta$ -cells in the islets of Langerhans (Okada et al., 1976). GABA is a major inhibitory neurotransmitter in the CNS, whereas its function in islets remains elusive. The expression of GABA<sub>A</sub> receptors on the glucagon producing  $\alpha$ -cells in islets (Rorsman et al., 1989) suggests that GABA may play a role in paracrine signaling within the islet. GAD has been identified as the 64-kD autoantigen of the  $\beta$ -cells (Baekkeskov et al., 1990), to which autoantibodies arise concomitant with  $\beta$ -cell destruction and the development of insulin-dependent diabetes mellitus (IDDM) (Baekkeskov et al., 1982, 1987; Atkinson et al., 1990). Furthermore, GAD in neurons is an autoantigen in stiff-man syndrome, a rare neurological disease characterized by a high coincidence with IDDM (Solimena et al., 1990). The

reasons for the unusual susceptibility of the GAD molecule to becoming an autoantigen are unclear, and their elucidation is a major goal. Neurons express two GAD proteins encoded by two distinct genes. The proteins differ mainly in the first 100 amino acids, but share extensive homology in the remainder of the molecules (Erlander et al., 1991).

We have identified two GAD proteins in pancreatic  $\beta$ -cells (Baekkeskov et al., 1990; Christgau et al., 1991). The larger 65-kD protein, which is expressed at low levels in rat islets, is hydrophilic and soluble throughout its lifetime and was shown to be identical to the larger GAD protein in brain (Christgau et al., 1991). This protein is now called GAD<sub>67</sub> (also called GAD-1 and previously GAD<sub>65</sub>). The smaller 64-kD protein, which is the major GAD protein in  $\beta$ -cells, was shown to be identical to the smaller GAD protein in brain, with regard to antigenic epitopes, size and charge (Christgau et al., 1991). A comparison of the primary structure of the smaller GAD protein in islets (Karlsen et al., 1991) and the smaller GAD protein in brain (Bu et al., 1992) shows that they are identical. This protein is now called GAD<sub>65</sub> (also called GAD-2 and previously GAD<sub>64</sub>). GAD<sub>65</sub> is also synthesized as a hydrophilic and soluble molecule but matures into a firmly membrane anchored protein in a process that seems to involve two steps (Christgau et al., 1991). The first step results in a hydrophobic molecule which is soluble or has a low membrane avidity. The second step results in membrane anchoring. Thus three distinct forms of

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1. *Abbreviations used in this paper:* GABA,  $\gamma$ -aminobutyric acid; GAD, glutamic acid decarboxylase; IDDM, insulin dependent diabetes mellitus; PIG, phosphatidyl inositol glycan; PIPLC, phosphatidyl inositol-specific phospholipase C; RP-HPLC, reverse phase high pressure liquid chromatography; S1, First cytosol fraction; S2, second cytosol fraction; SMS, stiff-man syndrome; TX-114, Triton X-114; WP, washed particle fraction.

GAD<sub>65</sub> can be detected in pancreatic  $\beta$ -cells, a hydrophilic soluble form, a hydrophobic form that is soluble or has a low membrane avidity, and a firmly membrane-bound form that can only be released from membranes by detergent, and thus has the characteristics of an intrinsic membrane protein. The maturation of GAD<sub>65</sub> does not involve detectable changes in size or charge, which suggests that membrane anchoring may be mediated by a small lipid (Christgau et al., 1991). Although antibodies in many diabetic and stiff-man syndrome patients recognize both GAD<sub>67</sub> and GAD<sub>65</sub> in their native configuration, the incidence of antibodies to GAD<sub>65</sub> is significantly higher than to GAD<sub>67</sub> (our unpublished results). Furthermore, GAD<sub>65</sub>, but not GAD<sub>67</sub>, possesses a primary structure epitope or epitopes that are recognized by stiff-man syndrome antibodies but not by IDDM antibodies (Baekkeskov et al., 1990). Thus GAD<sub>65</sub> has autoantigenic properties that clearly set it aside from GAD<sub>67</sub> and that may be related to its subcellular targeting to membrane compartments. GAD is an intracellular enzyme, but its exact subcellular localization is not known. Immunohistochemical staining and confocal microscopy analysis of neurons and  $\beta$ -cells showed colocalization of GAD with GABA and synaptophysin, a marker protein for synaptic vesicles (Reetz et al., 1991). Such studies have also demonstrated the localization of GAD and GABA to a vesicular compartment, which is clearly distinct from the insulin secretory granules in  $\beta$ -cells (Sorenson et al., 1991; Reetz et al., 1991) and which was tentatively identified as synaptic vesicles in neurons and synaptic like microvesicles in  $\beta$ -cells (Reetz et al., 1991). Immunoperoxidase EM of brain sections and immunogold EM of brain and islet cell homogenates using GAD antibodies however did not show GAD immunoreactivity in synaptic vesicles but rather localized GAD to the cytoplasm and in the proximity of synaptic vesicles in neurons and around membranes of pleomorphic microvesicles of tubular and cisternal elements, as well as throughout the cytoplasm in pancreatic  $\beta$ -cells (Wood et al., 1976; Reetz et al., 1991). Furthermore, GAD could not be recovered on synaptic vesicles or synaptic-like microvesicles nor in other membrane compartments after subcellular fractionation (Reetz et al., 1991). The EM analysis and the subcellular fractionation data are consistent with the localization of GAD<sub>67</sub> in the cytoplasm, but raise questions about the subcellular localization of the membrane bound GAD<sub>65</sub>.

In this report we have analyzed the subcellular localization of GAD<sub>65</sub> in intact islets by immunogold EM. Furthermore, we have characterized the hydrophobic membrane anchoring domain in the GAD<sub>65</sub> molecule and addressed the question of whether GAD<sub>65</sub> is modified by one or more of the lipid moieties which are known to mediate membrane anchoring of proteins, i.e., phosphatidylinositolglycan (PIG) polyisoprenyl groups, and/or fatty acids (Sefton and Buss 1987; Hancock et al., 1989; Casey et al., 1989). We present evidence that GAD<sub>65</sub> is anchored to the membrane of vesicles, which are likely to be the synaptic-like microvesicles in  $\beta$ -cells, and that membrane anchoring is achieved by a post-translational hydroxylamine-sensitive palmitoylation in the NH<sub>2</sub>-terminal region of the molecule where GAD<sub>65</sub> differs significantly from GAD<sub>67</sub>. The membrane anchoring is reversible, which may reflect the possibility of controlling the amount of the protein in membrane compartments.

## Materials and Methods

### Antibodies

S3, a sheep antiserum raised to purified rat brain GAD (Oertel et al., 1980), was a gift from I. Kopin (National Institutes of Health). The GAD6 mAb (Chang and Gottlieb, 1988) was a gift from D. Gottlieb (Washington University, St. Louis, MO). A polyclonal rabbit antibody (1266) raised against a synthetic peptide containing the carboxyl-terminal sequence of rat GAD<sub>67</sub> (Cys-Thr-Gln-Ser-Asp-Ile-Asp-Phe-Leu-Ile-Glu-Glu-Ile-Glu-Arg-Leu-Gly-Gln-Asp-Leu) (Wyborski et al., 1990) was a gift from J. S. Petersen (Hagedorn Research Laboratory, Gentofte, Denmark). Sera from IDDM patients with high titers of GAD autoantibodies have been described earlier (Baekkeskov et al., 1989).

### Isolation of Islets and Biosynthetic Labeling

Rat islets were used in all experiments described in this study except that biosynthetic labeling with <sup>3</sup>H-fatty acids was also carried out using human islets. Isolation of human and rat islets, maintenance in culture and radioactive labeling with [<sup>35</sup>S]methionine for 4 h was carried out as described (Baekkeskov et al., 1989). For biosynthetic labeling with fatty acids, islets were washed twice by centrifugation in DME containing 16 mM glucose and supplemented with 5 mM Na-pyruvate, 5 mg/ml fatty acid free BSA, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and nonessential amino acids (FA medium). Islets were labeled for 4 h at 37°C in the same medium containing either (9, 10) <sup>3</sup>H-palmitic acid or (9, 10) <sup>3</sup>H-myristic acid (50–60 Ci/mmol, Amersham Corp., Arlington Heights, IL) at 800  $\mu$ Ci/ml. In experiments to analyze effect of emetine in labeling experiments, islets were preincubated with 10  $\mu$ M emetine followed by [<sup>35</sup>S]methionine labeling for 4 h in the presence of 10  $\mu$ M emetine. Similarly in experiments with mevinolin (a kind gift from Dr. A. W. Alberts, Merck, Sharp, and Dohme Research Laboratories, Rahway, NJ), islets were preincubated for 1 h with 10, 20, 30, 40, or 50  $\mu$ M mevinolin and then labeled for 4 h with [<sup>35</sup>S]methionine in the presence of the same concentrations of mevinolin. For biosynthetic labeling with <sup>3</sup>H-mevalonic acid, islets were preincubated for 1 h with 50  $\mu$ M mevinolin in FA medium and labeled with <sup>3</sup>H-mevalonolactone (36 Ci/mmol, New England Nuclear DuPont Co., Wilmington, DE) at 250  $\mu$ Ci/ml for 4 h. Islets were also labeled with <sup>3</sup>H-myoinositol (90.5 Ci/mmol, Amersham Corp.), <sup>3</sup>H-ethanolamine (40 Ci/mmol, Amersham Corp.) and <sup>3</sup>H-glucosamine (32 Ci/mmol, Amersham Corp.) for 6 h in RPMI 1640 containing 16 mM glucose and supplemented with 1% FCS, 0.5% human serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and isotope at 200  $\mu$ Ci/ml. Labeled islets were harvested by centrifugation, washed twice in nonradioactive medium and once in 20 mM Hepes, pH 7.4, 150 mM NaCl and 10 mM benzamidine/HCl, and then either immediately processed for homogenization and isolation of soluble and particulate fractions or snap frozen and stored in aliquots at –80°C.

### Expression of GAD<sub>65</sub> in Sf9 Insect Cells

Recombinant GAD<sub>65</sub> baculovirus vectors were constructed by ligating a 2.4-kb EcoRI fragment of rat GAD<sub>65</sub> cDNA clone pGAD-92 (a kind gift from Dr. A. Tobin, UCLA) into the EcoRI site of the baculovirus transfer vector pVL1392 (Invitrogen, San Diego, CA). Clone pGAD-515 containing the sense orientation for GAD<sub>65</sub> was used in a calcium phosphate cotransfection with wild-type *Autographa californica* nuclear polyhedrosis virus. Recombinant viruses were then isolated as described (Summers and Smith, 1987). For radiolabeling of GAD<sub>65</sub> in insect cells, *Spodoptera frugiperda* Sf9 cells were infected with recombinant baculovirus. 48 h later the cells were labeled for 4 h with either <sup>3</sup>H-palmitate, <sup>3</sup>H-myristate, <sup>3</sup>H-mevalonolactone at 800  $\mu$ Ci/ml, or <sup>35</sup>S-methionine at 80  $\mu$ Ci/ml as described above for islets, except that labeling was in Grace medium.

### Fractionation of Islets and Sf9 Cells

Separation of soluble and membrane-bound fractions was carried out by two methods described in detail previously (Christgau et al., 1991; Baekkeskov et al., 1990). The first method involves homogenization in an isotonic Hepes/sucrose buffer (10 mM Hepes/NaOH, pH 7.4, 0.25 M sucrose, 10 mM benzamidine/HCl, 0.1 mM p-chloromercuribenzenesulfonic acid and 0.25% Trasylol) (Christgau et al., 1991) followed by ultracentrifugation at

100,000 g or higher for 1 h to separate a crude particulate and a cytosol (S1) fraction. The crude particulate fraction is then resuspended in Hepes buffer A (10 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 10 mM benzamidine/HCl, 0.25% Trasylol, 0.1 mM P-chloromercuribenzenesulfonic acid and 0.1 mM Na<sub>3</sub>VO<sub>4</sub>) followed by ultracentrifugation at 100,000 g or higher for 1 h resulting in a new cytosol fraction, S2, and a washed particulate fraction, WP. The S1 fraction prepared by this method contains mainly GAD<sub>67</sub> and the hydrophilic soluble form of GAD<sub>65</sub>. The crude particulate fraction contains both a hydrophobic firmly membrane anchored GAD<sub>65</sub> and hydrophobic GAD<sub>65</sub> which is either soluble or has a low membrane avidity. The two hydrophobic GAD<sub>65</sub> forms separate into the WP and S2 fractions, respectively (Christgau et al., 1991). The second method uses homogenization in a hypotonic Hepes buffer (10 mM Hepes/NaOH, pH 7.4, 1 mM EGTA, 1 mM MgCl<sub>2</sub>, 1 mM aminoethylisothiuronium bromide hydrobromide, and 0.2 mM pyridoxal 5'-phosphate (Hepes buffer B) (Baekkeskov et al., 1990), followed by ultracentrifugation at 100,000 g or higher. The hypotonic conditions result in release of most of the hydrophobic, soluble, or low membrane avidity GAD<sub>65</sub> form into the cytosol and combine in one fraction the soluble forms of GAD<sub>65</sub>. The membrane fraction contains the firmly membrane-anchored hydrophobic GAD<sub>65</sub> form which can only be released by detergents.

Proteins were extracted from the particulate fractions with 1% Triton X-114 (TX-114) in either Hepes buffer A or B, as described (Baekkeskov et al., 1990; Christgau et al., 1991).

### TX-114 Partitioning Assays

TX-114 detergent phase separations of detergent extracts of particulate fractions or of soluble fractions after addition of TX-114 were performed according to Bordier (1981) (Baekkeskov et al., 1989). For comparative analyses of the amphiphilic properties of GAD<sub>65</sub> in different fractions, buffer compositions were adjusted to obtain identical conditions in each fraction.

### Experiments with Phosphatidyl Inositol-specific Phospholipase C

The phosphatidyl inositol-specific phospholipase C (PIPLC) used for these experiments was either from *Bacillus thuringiensis* (a kind gift from Dr. M. Low, The Rockefeller University, New York) or *Bacillus cereus* (Boehringer Mannheim Corp., Indianapolis, IN). Islets were homogenized in isotonic Hepes buffer and the P-100 fraction washed twice by resuspension and ultracentrifugation followed by resuspension and incubation for 30, 60, or 120 min at 0, 13, 24, and 37°C in the presence or absence of PIPLC at concentrations ranging from 125 mU/ml to 5 U/ml. Experiments were performed using three different buffer compositions for washings of the P-100 fraction and the incubations with and without PIPLC. Hepes buffer C was 10 mM Hepes/NaOH, pH 7.4, supplemented with 0.25% Trasylol. Hepes buffer D was 10 mM Hepes/NaOH, pH 7.4, supplemented with 10 µg/ml pepstatin A, 20 µg/ml leupeptin, 10 µg/ml antipain, and 0.01% Trasylol. Buffer E was 10 mM Tris/HCl, pH 7.5, 0.25 M sucrose, 5 mM PMSF, 5 mM iodoacetate, 1 mM leupeptin, and 100 µM N-α-p-tosyl-L-lysine chloromethyl ketone. Hepes buffer A was used for control incubations.

### Immunochemical Analysis and Gelelectrophoresis

SDS-PAGE, immunoprecipitation, and immunoblotting were performed as described (Baekkeskov et al., 1989, 1990; Christgau et al., 1991).

### Chemical Analysis of the Fatty Acid Group

For reverse phase high pressure liquid chromatography (RP-HPLC) analysis of covalently bound fatty acids, <sup>3</sup>H-palmitate-labeled GAD<sub>65</sub> was excised from SDS-gels and the gel pieces were incubated for 2 h at 30°C in 1.5 M NaOH in 20% methanol to release fatty acids followed by pentane extraction to remove neutral lipids. The reaction mixture was acidified with HCl and fatty acids extracted into pentane. The free fatty acids isolated from GAD<sub>65</sub> and cold standards were derivatized to their corresponding phenacyl esters according to Wood and Lee (1983), and analyzed on an Altex C-18 ultrasphere reverse phase HPLC column. Bound material was eluted with a linear gradient of 75–100% acetonitrile. Absorbance at 242 nm and cpm were measured in the eluted fractions. For RP-HPLC analysis of total fatty acids in Sf9 cells, the membrane fraction was extracted with chloroform methanol, hydrolyzed, and analyzed as described above. Peaks were identified by comparison with the retention time for cold fatty acid standards carried along through the derivatization procedure.

### Tryptic Cleavage of GAD

TX-114 detergent phase purified particulate and soluble fractions prepared in hypotonic Hepes buffer were diluted to 1% TX-114 and incubated with and without trypsin as described (Baekkeskov et al., 1990) followed by a renewed phase separation and analysis of aqueous and detergent phases by immunoblotting. To analyze the ability of trypsin to release GAD<sub>65</sub> from membranes, aliquots of P-100 were prepared, washed two times in Hepes buffer B, resuspended in 200 µl of the same buffer, and incubated for 1 h at 25°C in the presence or absence of 4.0 U trypsin (Sigma Chem. Co., St. Louis, MO). The trypsin digestion was terminated by placing the samples on ice and adding 1/10 vol of 10 mM Hepes/NaOH, pH 7.5, 5 mM benzamidine/HCl, 5 mM EDTA, 5 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, and 1/10 vol of 1 mg/ml soya bean trypsin inhibitor in water. Particulate material was sedimented by ultracentrifugation at 265,000 g. Released material and particulate extracts were subjected to TX-114 phase separation followed by Western blot analysis of GAD in the different fractions.

### Spontaneous Release of GAD<sub>65</sub> from Membrane Fractions

Islets were homogenized in 10 mM Hepes, 150 mM NaCl, pH 7.4 (Hepes buffer F). The P-100 particulate fraction was washed twice in the same buffer and separated into aliquots. Membrane aliquots were resuspended in either Hepes buffer F or in the same buffer supplemented with 0.1 mM PCMBs, 5 mM EDTA, 0.25% trasylol, 5 mM NaF, 10 mM benzamidine/HCl, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, pH 7.4, and incubated for 5, 15, 45, and 75 min at 25°C, respectively, followed by centrifugation at 265,000 g for 30 min and analysis of released material by Western blotting.

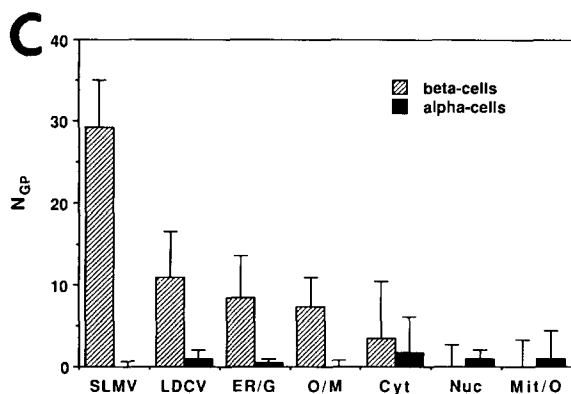
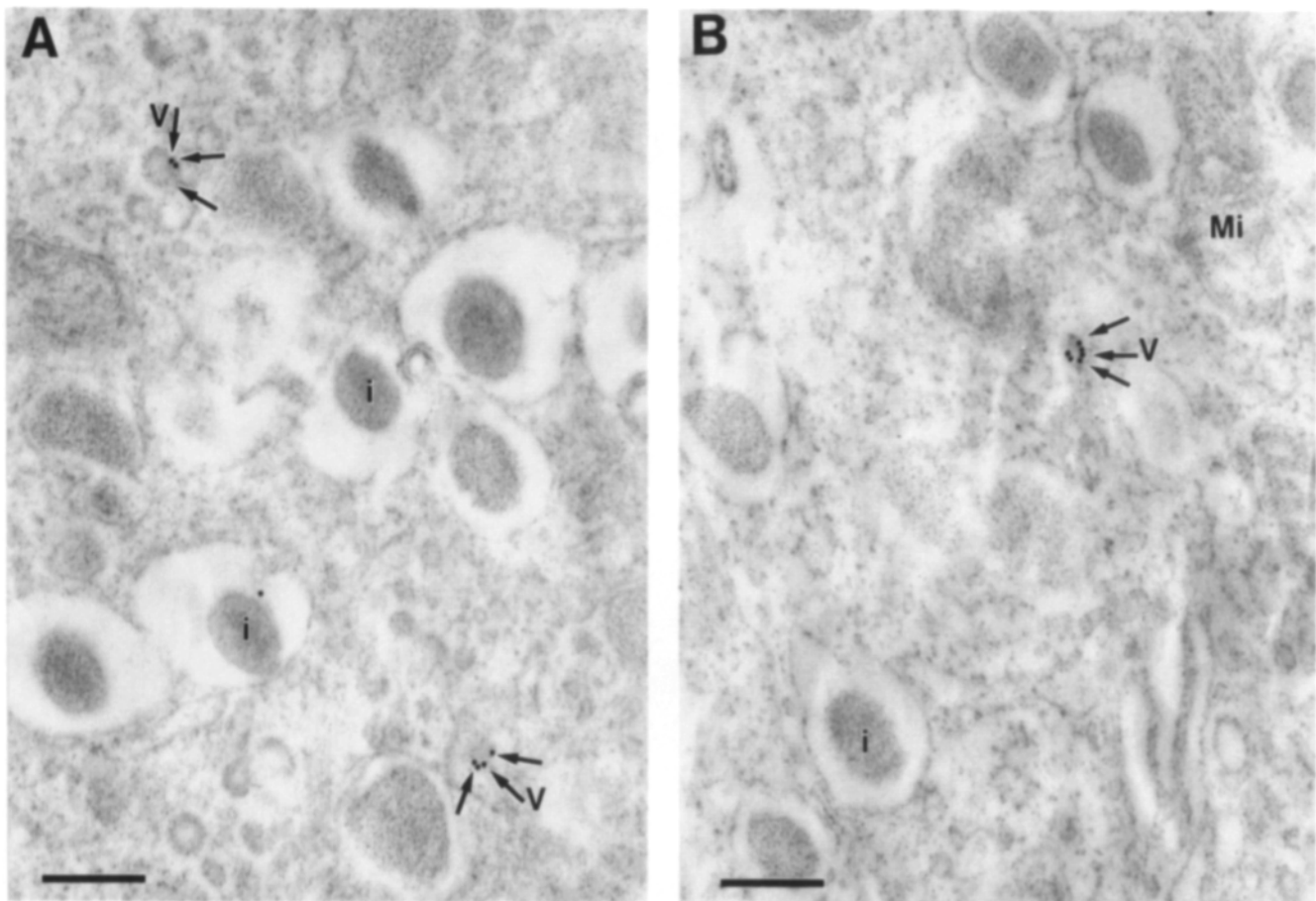
### Electron Microscopy

Immunogold electronmicroscopy was performed on neonatal rat islets, which had been maintained in culture for 3–5 d. Islets were fixed for 6 h in 4% paraformaldehyde, 0.1% glutaraldehyde in 100 mM cacodylate buffer containing 0.2 mM CaCl<sub>2</sub>, and 4% sucrose. Islets were postfixed for 15 min in 1% OsO<sub>4</sub>, 1.5% ferricyanide in 0.1 M 2,4,6-trimethylpyridine and for 30 min in 2% aqueous uranyl acetate. Dehydration and embedding in LR White resin (medium hardness; Polysciences Inc., Warrington, PA) was performed as described (Newman et al., 1983). 50-nm sections were cut on a Ultracut E microtome (Reichert Jung, Deerfield, IL) and placed on grids. Antibody incubation was for 18 h with the GAD antiserum 1266 or S3 (dilution 1:100) followed by incubation with appropriate 10-nm gold-conjugated secondary antibodies (E-Y Labs., San Mateo, CA). Control grids were stained with normal sheep or rabbit serum followed by the secondary antiserum or with secondary antiserum only. Grids were coated with formvar and viewed on a JEOL-100C transmission electron microscope at 60 kV. β-cells as well as non-β-cells (α-cells, exocrine cells) were viewed on each grid and subjected to quantitative analysis by counting the gold particles. α-cells and exocrine cells did not differ significantly in total gold particle counts and α-cells were selected as control cells for further subcellular analysis. Gold particles were counted in 19 β-cells on 19 different grids representing 4 different islet isolations and in 7 adjacent α-cells stained with the 1266 antibody. 1821 gold particles were detected in the β-cells, giving 95.8 ± 17 (mean ± SD) particles per β-cell. The α-cell counts revealed 226 particles or 32.3 ± 4.7 particles per α-cell. As a control six β-cells (227 particles, 41.3 ± 2.5 per β-cell) and five adjacent α-cells (165 particles, 33 ± 4.7 per α-cell) were counted on six grids stained with normal rabbit serum. Cells were divided into the following subcellular areas during the quantitative analysis: small vesicles of diameter 30–60 nm (49.6 ± 17.6, mean ± SD), ER/Golgi, other membranes (including plasma membranes and unidentified membranes), cytoplasm, nuclei, large dense core vesicles (insulin or glucagon secretory vesicles), mitochondria/other (other = unidentified subcellular structures). The mean values for each fraction obtained with the 1266 serum were corrected by subtraction of mean values obtained with the nonimmune serum and the pooled standard deviations calculated.

### Results

#### GAD<sub>65</sub> Is Localized to the Membrane of Small Vesicles

To analyze the subcellular localization of GAD<sub>65</sub>, sections of fixed and embedded rat islets were sectioned and sub-



**Figure 1.** Electron micrographic analysis of GAD expression in pancreatic islet cells. (*A* and *B*) Micrographs of  $\beta$ -cell areas showing the localization of GAD by an immunogold labeling technique. The sections shown in *A* and *B* were stained with the S3 antiserum to GAD. Some microvesicles with a clear core (*V*) show GAD staining in the membrane. Insulin-containing secretory granules (*i*) are characterized by a typical halo around a dense granule core. *Mi*, mitochondrion. (*C*) Quantitative analysis of the localization of GAD in different subcellular areas of  $\beta$  (black bars) and  $\alpha$ -cells (shaded bars) on electronmicrographs subjected to immunogold labeling using the 1266 antiserum to GAD.  $N_{GP}$  is the corrected number of gold particles obtained by subtracting nonimmune serum values from those produced with the GAD specific 1266 antiserum as described in Materials and Methods. SD is indicated on the bars. *SLMV*, small vesicles 30–60 nm in diam; *LDCV*, large dense core vesicles which secrete insulin or glucagon; *ER/G*, ER and Golgi membranes; *O/M*, other nonidentified membranes and plasma membranes; *Cyt*, Cytoplasm; *Nuc*, Nuclei; *Mit/O* Mitochondria and nonidentified subcellular structures. Bars, 240 nm.

jected to immunogold staining using both the S3 antiserum, which has a high immunoreactivity to GAD<sub>65</sub> but reacts only weakly with GAD<sub>67</sub> in immunoprecipitation experiments and on Western blots (results not shown), and the 1266 antiserum, which recognizes both forms (Christgau et al., 1991). The specificity of EM immunogold labeling of fixed and embedded tissue with both antisera was high and the sensitivity low (Fig. 1). The predominant immunolabeling for GAD with either antiserum was in the membrane of small vesicles (30–60 nm in diam) in  $\beta$ -cells (Fig. 1, *A–C*). The

specificity of this localization is illustrated by the number of gold particles detected in this compartment in  $\beta$ -cells immunolabeled with the 1266 antiserum ( $29.7 \pm 6.6$  particles/cell, mean  $\pm$  SD) which was  $\sim 60$ -fold higher than the labeling of comparable small vesicles in  $\alpha$ -cells ( $0.4 \pm 0.7$  particles/cell), and 60–75-fold higher than nonspecific labeling of small vesicles in  $\beta$ - and  $\alpha$ -cells produced with a nonimmune serum ( $0.5 \pm 0.8$  particles/cell and  $0.4 \pm 0.5$  particles/cell, respectively). The gold particles in  $\beta$ -cells labeled with nonimmune serum and in  $\alpha$ -cells labeled with

either GAD antiserum or nonimmune serum were concentrated in the cytoplasm, mitochondria, and nuclear fractions. The results of a quantitative analysis of electronmicrographs immunolabeled with the 1266 antiserum and corrected for background immunolabeling with nonimmune serum (Fig. 1 C) indicate that ~50% of the specific immunolabeling for GAD in  $\beta$ -cells is localized to the membrane of the small vesicles, 18% is found in insulin secretory granules, 14% in ER/Golgi membranes, 12% in other membranes (unidentified membranes and plasma membranes), and 6% in the cytoplasm. The low level of specific GAD immunolabeling in the cytoplasm of  $\beta$ -cells is consistent with the low level of cytosolic GAD<sub>67</sub> protein in these cells and with the majority of the GAD<sub>65</sub> protein being membrane associated (Christgau et al., 1991). The specific GAD immunolabeling in insulin granules was detected in the perigranular space but not in the dense insulin core. The gold particles sometimes formed characteristic circular structures of 30–60 nm in diameter just inside the insulin secretory granule membrane (results not shown) suggesting that they represent small vesicles that have fused with the insulin granule. This result, which merits further investigation, suggests that the small GAD-positive vesicles can fuse with and perhaps release GABA into the insulin secretory granules. The specific GAD immunolabeling in all compartments was restricted to  $\beta$ -cells. Thus the number of gold particles in  $\alpha$ -cells (Fig. 1 C) and exocrine cells (results not shown) was not significantly different from background immunolabeling with nonspecific serum, which is consistent with the  $\beta$ -cell specific expression of GAD (Okada et al., 1976; Baekkeskov et al., 1990; Sorensen et al., 1991). The results provide evidence that GAD<sub>65</sub> is predominantly localized to the membrane of microvesicles in the pancreatic  $\beta$ -cell. The small vesicles are clearly distinct from the insulin granules in those cells (Fig. 1) and are likely to be the synaptic-like microvesicles containing GABA which have been described in  $\beta$ -cells (Reetz et al., 1991). This result raises questions of the mechanism by which this subcellular localization is achieved. The following sections characterize the membrane anchoring of GAD<sub>65</sub>.

#### ***Palmitoylation Distinguishes Membrane-bound and Soluble Forms of GAD<sub>65</sub>***

Depending on the conditions by which islets of Langerhans are homogenized and separated into soluble and particulate fractions, hydrophilic soluble GAD<sub>65</sub> (S1-form) and hydrophobic soluble or low membrane avidity GAD<sub>65</sub> (S2-form) can either be released simultaneously (in hypotonic Hepes homogenization buffer), or sequentially (in isotonic Hepes/sucrose homogenization buffer followed by resuspension of a crude particulate fraction in an isotonic Hepes buffer and resedimentation of membranes to form WP). The hydrophobic membrane-anchored GAD<sub>65</sub> (WP-form) can, in both conditions, only be released from the particulate fraction by detergent (Christgau et al., 1991 and results not shown). Two-dimensional gel electrophoretic analyses of the membrane-anchored and soluble GAD<sub>65</sub> forms has not revealed any differences in size or charge between the different forms (Christgau et al., 1991), suggesting that the hydrophobic modification and membrane anchoring is mediated by a fatty acid or a small neutral lipid.

To assess the possibility that GAD<sub>65</sub> contains fatty acids

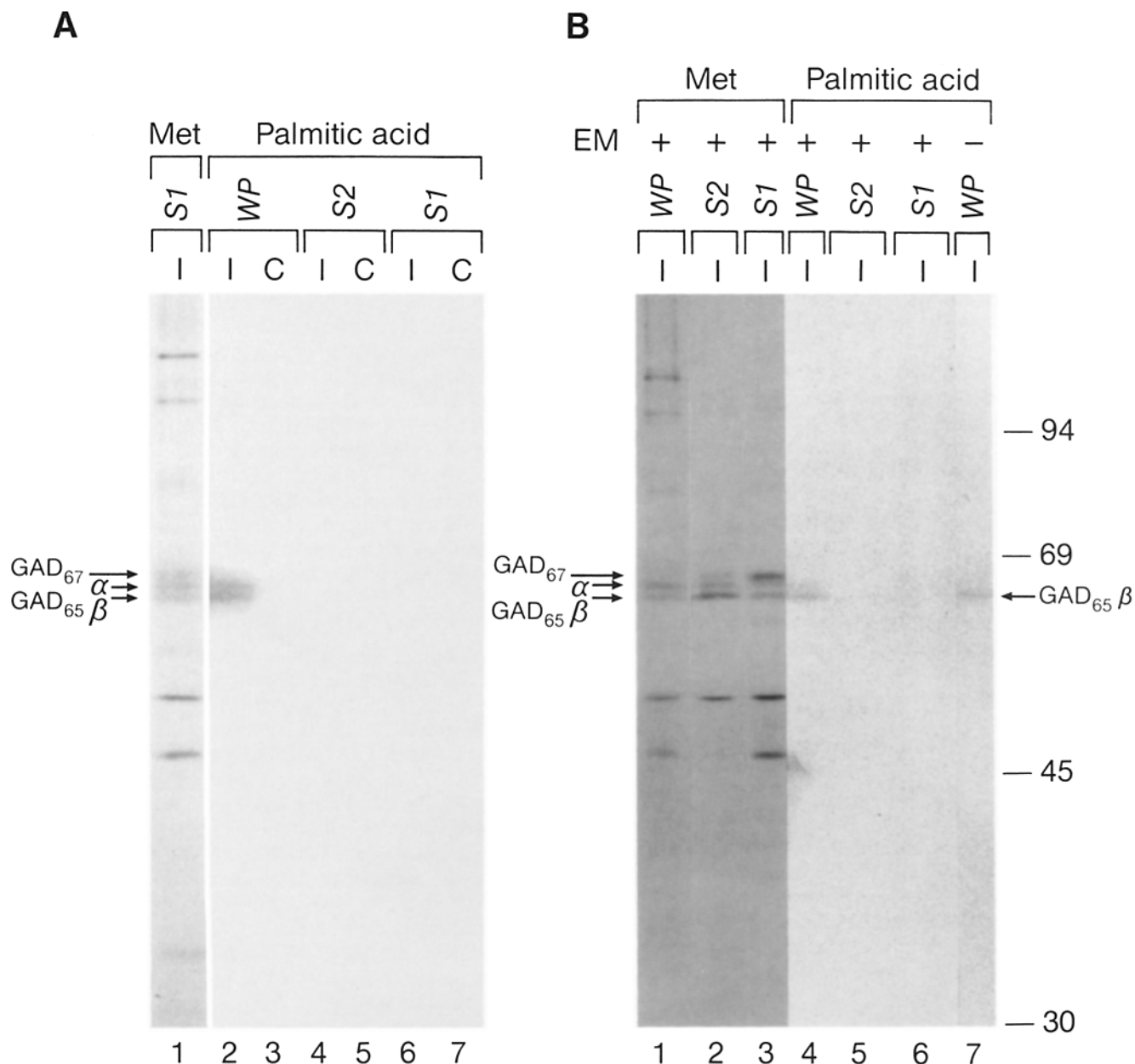
either covalently linked to the protein backbone or as a part of a larger lipid structure, human and rat islets were labeled for 4 h with either <sup>3</sup>H-palmitic acid or <sup>3</sup>H-myristic acid at the same specific activity. Islets were homogenized in isotonic conditions and the two cytosolic fractions, S1 and S2, and the washed WP were prepared. GAD<sub>65</sub> was isolated by immunoprecipitation and analyzed by SDS-PAGE and fluorography (Fig. 2). The incorporation of radioactivity was detected in GAD<sub>65</sub> immunoprecipitated from detergent lysates of whole human islets (results not shown) and only in the washed membrane fractions but not the cytosolic S1 and S2 fractions of rat islets labeled with <sup>3</sup>H-palmitic acid (Fig. 2). Incorporation of radioactivity into GAD<sub>65</sub> was not detected in experiments with <sup>3</sup>H-myristic acid (results not shown). The absence of label in background bands and in soluble forms of GAD<sub>65</sub> in the immunoprecipitates from <sup>3</sup>H-palmitic acid-labeled material (Fig. 2) suggested that the incorporation of tritium into membrane-bound GAD<sub>65</sub> was not due to conversion of label to radioactive amino acids. Thus the results suggest that palmitic acid itself or a lipid derivative of palmitate is covalently linked to the membrane anchored but not the soluble forms of GAD<sub>65</sub> and is involved in the membrane anchoring of the enzyme.

#### ***Palmitoylation of GAD<sub>65</sub> Is a Posttranslational Event***

Palmitoylation of some cellular proteins is greatly reduced when protein synthesis is inhibited, indicating that acylation in these cases is cotranslational (Magee and Courtneidge, 1985; Olson et al., 1985). In contrast, acylation of other proteins, including p21 N-ras (Magee et al., 1987), GAP 43 (Skene and Virag, 1989), and others (Olson and Spizz, 1986), is unaffected by these inhibitors, showing that acylation of these proteins is posttranslational. To assess whether the incorporation of radioactivity into membrane-anchored GAD<sub>65</sub> in the presence of <sup>3</sup>H-palmitic acid is dependent on protein synthesis, we analyzed the effect of emetine, an inhibitor of protein biosynthesis (Fig. 2 B). The presence of 10  $\mu$ M emetine did not inhibit incorporation of radioactivity into membrane-anchored GAD<sub>65</sub> in labeling experiments with <sup>3</sup>H-palmitic acid (Fig. 2 B, compare lanes 4 and 7), despite inhibiting [<sup>35</sup>S]methionine incorporation >90%. This result shows that the labeling of the membrane-anchored GAD<sub>65</sub> in the presence of <sup>3</sup>H-palmitic acid is a posttranslational event independent of protein synthesis and excludes the possibility that the protein is labeled by biosynthetic incorporation of <sup>3</sup>H amino acids derived from palmitate. The data do not, however, exclude the possibility that GAD<sub>65</sub> is modified posttranslationally by a lipid derivative of palmitate.

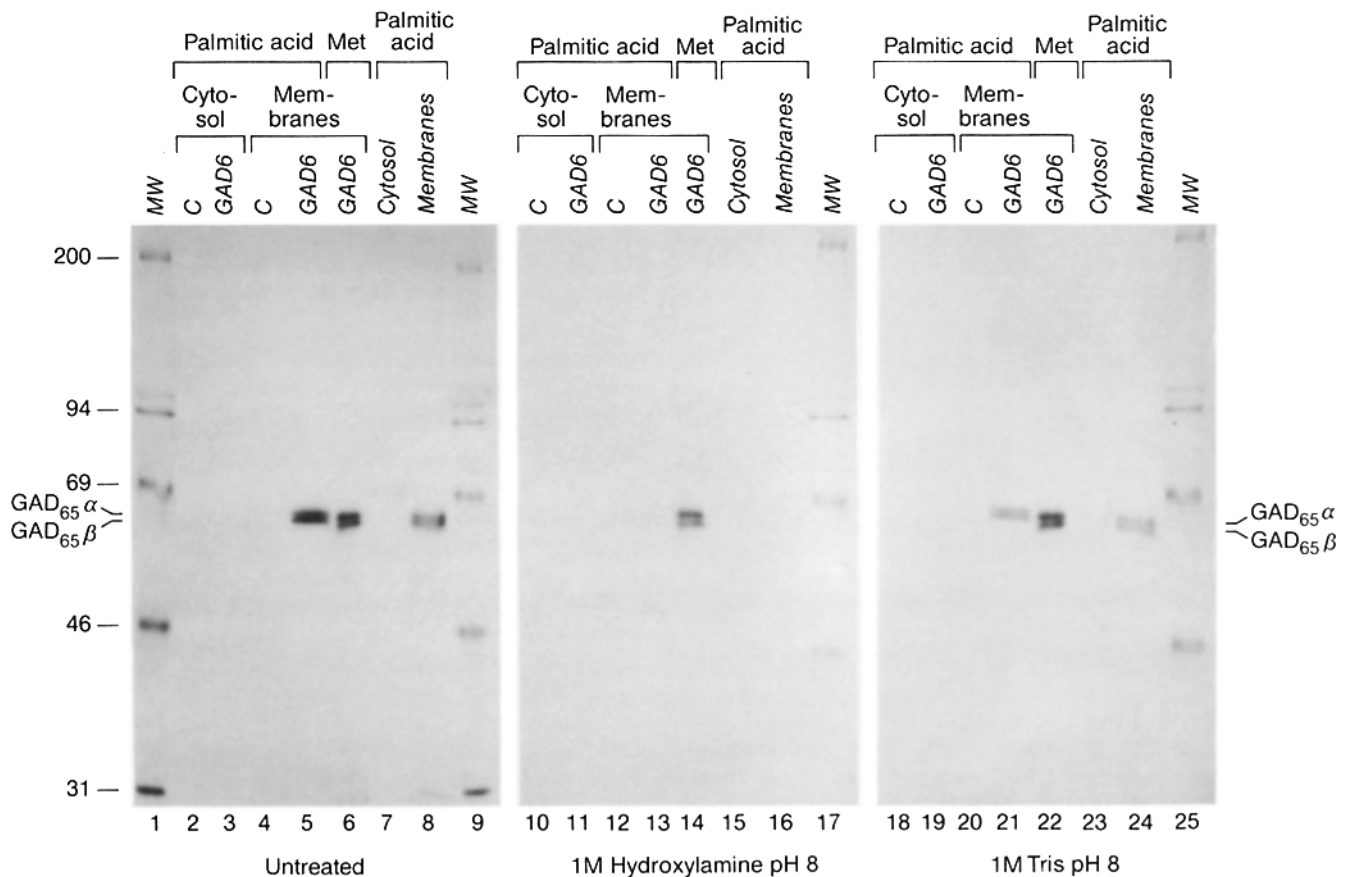
#### ***GAD Is Palmitoylated in Sf9 Insect Cells***

Sf9 insect cells have been shown to correctly modify expressed proteins with both palmityl (Lanford, 1988), myristyl (Gheysen et al., 1989), and isoprenyl moieties (Khosravi-Far et al., 1991). Since GAD<sub>65</sub> is a rare protein in pancreatic  $\beta$ -cells (Baekkeskov et al., 1989), and since the autoradiographic detection of <sup>3</sup>H is very insensitive, we assessed whether recombinant GAD<sub>65</sub> expressed at high levels in Sf9 cells could be used to further characterize <sup>3</sup>H-palmitate-labeled GAD<sub>65</sub>. GAD<sub>65</sub> was distributed in soluble and membrane-bound fractions in Sf9 cells, much as in pancreatic



**Figure 2.** Posttranslational incorporation of palmitic acid into membrane-bound but not soluble forms of GAD<sub>65</sub>. **(A)** Radioactive labeling of membrane bound GAD<sub>65</sub> in the presence of <sup>3</sup>H-palmitic acid. Rat islets were labeled in the presence of <sup>3</sup>H-palmitic acid for 4 h and subjected to homogenization in isotonic conditions to prepare a cytosolic (S1) and a crude membrane fraction. The crude membrane fraction was washed by resuspension and centrifugation resulting in a supernatant (S2) containing mainly cytosolic proteins and WP fractions. The S1 fraction (lanes 6 and 7), was concentrated fivefold and subjected to immunoprecipitation together with 2/3 of the S2 (lanes 4 and 5) and 1/3 of the WP fraction (lanes 6 and 7) to obtain similar levels of GAD<sub>65</sub> in each fraction. Immunoprecipitation was performed first with control serum (C) (lanes 3, 5 and 7) and then with IDDM serum (I) (lanes 2, 4, and 6). Immunoprecipitates were analyzed by SDS-PAGE in modified Laemmli buffers followed by fluorography. A short exposure to visualize [<sup>35</sup>S]methionine-labeled GAD<sub>67</sub> and GAD<sub>65</sub> components immunoprecipitated from the S1 fraction of 1,000 islets is shown in lane 1. Note that GAD<sub>65</sub> splits into two components α and β, which have been shown to be identical with regard to all parameters analyzed except mobility on SDS-PAGE. **(B)** Effect of emetine on incorporation of radioactivity into membrane-bound GAD<sub>65</sub> in the presence of palmitic acid. Rat islets were preincubated in FA medium either with or without 10 nM emetine for 1 h and then labeled with <sup>3</sup>H-palmitic acid in the same medium for 4 h. Aliquots of rat islets were incubated in parallel with or without emetine and labeled with [<sup>35</sup>S]methionine. S1, S2, and WP fractions were prepared, immunoprecipitated with IDDM serum (I), and analyzed by SDS-PAGE in modified Laemmli buffers and fluorography. Fluorography shown for [<sup>35</sup>S]methionine-labeled proteins was for 14 d with emetine (lanes 1, 2, and 3), at which time lanes without emetine were completely black (not shown). Fluorography for <sup>3</sup>H-labeled proteins with (lanes 4–6) and without emetine (lane 7) was for 3 mo. Note that in this experiment the GAD<sub>65</sub> β component is more pronounced than α in lanes 2, 3, 4, and 7. The position of molecular mass markers is indicated in kilodaltons.





**Figure 3.** Cellular localization and hydroxylamine sensitivity of  $^3\text{H}$  palmitate labeled  $\text{GAD}_{65}$  in Sf9 cells. Sf9 cells were infected with baculovirus containing the rat  $\text{GAD}_{65}$  cDNA and grown in the presence of either [ $^{35}\text{S}$ ]methionine or  $^3\text{H}$ -palmitic acid and separated into a cytosolic and a membrane fraction after homogenization in hypotonic conditions. Cytosol and membrane fractions from  $^3\text{H}$ -palmitic acid-labeled islets were immunoprecipitated with either the GAD6 mAb or an irrelevant mAb (C). Immunoprecipitates were divided into three equal size aliquots and analyzed in triplicate by SDS-PAGE (lanes 2–5, 10–13, and 18–21) in parallel with an aliquot of nonimmunoprecipitated cytosol and membrane fractions of  $^3\text{H}$ -palmitate-labeled material (lanes 7, 8, 15, 16, 23, and 24) and an immunoprecipitate of a [ $^{35}\text{S}$ ]methionine-labeled membrane fraction (lanes 6, 14 and 22). The triplicate gels were either untreated or incubated in either 1 M hydroxylamine, pH 8, or 1 M Tris, pH 8, before processing for fluorography. Molecular mass markers indicated in kilodaltons are shown in lanes 1, 9, 17, and 25.

$\beta$ -cells. Again, as in  $\beta$ -cells, membrane-bound  $\text{GAD}_{65}$  behaved like an intrinsic membrane protein, which could only be removed by detergent and not by salt, EDTA, or basic pH treatment (results not shown). Consistent with the results in  $\beta$ -cells, incorporation of radioactivity from  $^3\text{H}$ -palmitate was detected in membrane-bound but not soluble  $\text{GAD}_{65}$  in Sf9 cells (Fig. 3).

#### Palmitoylation of $\text{GAD}_{65}$ Is Hydroxylamine Sensitive

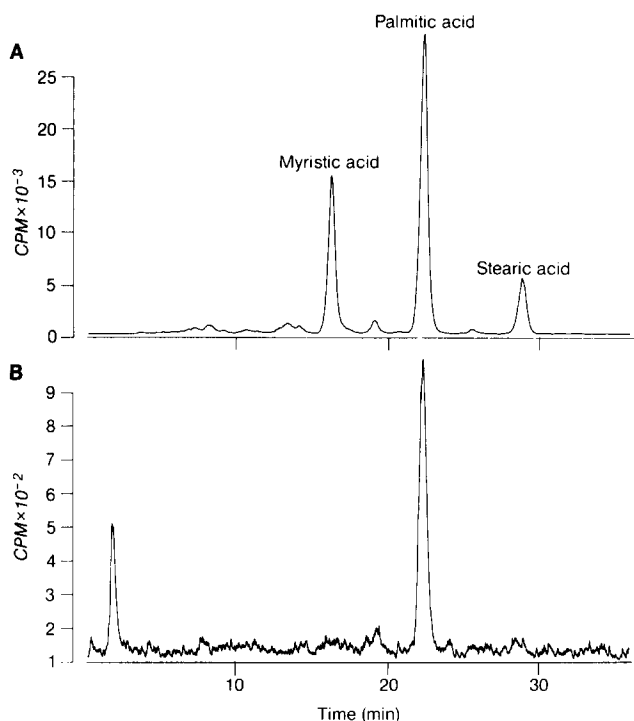
Hydroxylamine at pH 8.0 is used as a method for a gentle removal of fatty acid from proteins (Omary and Trowbridge, 1981; Magee et al., 1984) and can be used as a diagnostic test of a thioester- or ester-linked fatty acid. Incubations of  $^3\text{H}$ -palmitic-labeled membrane-bound  $\text{GAD}_{65}$  on SDS-polyacrylamide gels with hydroxylamine efficiently removed the radioactivity (Fig. 3, lanes 13 and 16) without loss of [ $^{35}\text{S}$ ]methionine-labeled  $\text{GAD}_{65}$  (Fig. 3, lane 14).

The fatty acid composition of the  $^3\text{H}$ -palmitate-labeled Sf9 cells was analyzed to assess whether palmitate had been converted to other fatty acids during the labeling period. Fatty acids were extracted from a membrane fraction prepared from the cells which contained 91% of the incorpo-

rated label. The fatty acids were derivatized and analyzed by reverse phase high performance liquid chromatography (RP-HPLC). Fatty acid standards were cochromatographed to allow the identification of extracted fatty acids. 51% of the incorporated label in Sf9 cells was found as palmitic acid, 26% was found as myristic acid, 10% was found as stearic acid, and 13% was found as minor unidentified peaks. (Fig. 4 A). The label incorporated into  $\text{GAD}_{65}$  was identified by excising radiolabeled  $\text{GAD}_{65}$  from gels, followed by hydrolysis under alkaline conditions. The released fatty acids were analyzed by RP-HPLC and identified by cochromatography with standards. Only palmitic acid was detected in  $\text{GAD}_{65}$  excised from gels (Fig. 4 B). Thus the incorporation of radioactivity into membrane-bound  $\text{GAD}_{65}$  in the presence of  $^3\text{H}$  palmitic acid represents palmitoylation of the protein and not incorporation of a palmitate-derived fatty acid confirming that the acylation of  $\text{GAD}_{65}$  is specific for palmitic acid.

The analyses of membrane-bound and soluble forms of  $\text{GAD}_{65}$  suggested that the hydrophobic soluble and hydrophobic membrane-bound  $\text{GAD}_{65}$  represent differential stages of a two-step posttranslational modification with hydrophobic residues. Thus, attachment of the first hydrophobic resi-





**Figure 4.** Identification of palmitic acid as the lipid attached to GAD<sub>65</sub>. (A) Reverse phase HPLC analysis of <sup>3</sup>H-labeled fatty acids isolated from <sup>3</sup>H-palmitic acid-labeled Sf9 cells. The positions of myristate, palmitate, and stearate were identified by cochromatography with standards. (B) Analysis of <sup>3</sup>H-labeled fatty acids isolated from GAD<sub>65</sub> purified from <sup>3</sup>H-palmitic acid-labeled Sf9 cells. The palmitate peak was identified by cochromatography with standards. The peak in the beginning of the chromatogram is nonderivatized fatty acids.

due would result in soluble hydrophobic GAD<sub>65</sub> and then palmitoylation of this form would result in the membrane-anchored form.

To examine the possibility that the GAD<sub>65</sub> autoantigen in  $\beta$ -cells is polyisoprenylated and that this modification is responsible for the hydrophobicity of the S2 form, we tested whether <sup>3</sup>H-mevalonate was incorporated into GAD<sub>65</sub> in either rat islet cells or Sf9 cells. The results of those experi-

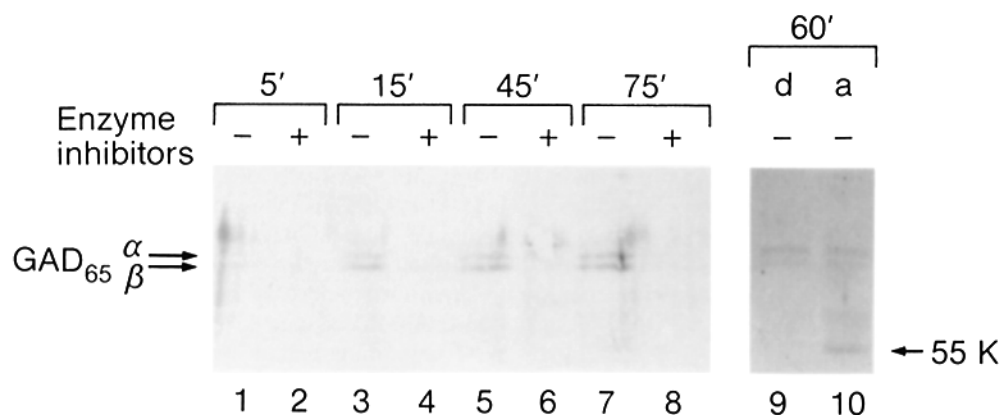
ments were negative, suggesting that a polyisoprenyl moiety is not a part of the hydrophobic modification and membrane anchoring of GAD<sub>65</sub> (data not shown). Mevinolin, an inhibitor of polyisoprenyl synthesis, also inhibited protein synthesis in islet cells, but did not inhibit hydrophobic modification and membrane anchoring of GAD<sub>65</sub> significantly. The results are consistent with the absence of the known COOH-terminal consensus sequences for polyisoprenylation, CAAX, CC, or CXC (Hancock et al., 1989; Khosravi-Far et al., 1991) in GAD<sub>65</sub> (Erlander et al., 1991).

### Spontaneous and Time-dependent Release of GAD<sub>65</sub> from Membranes

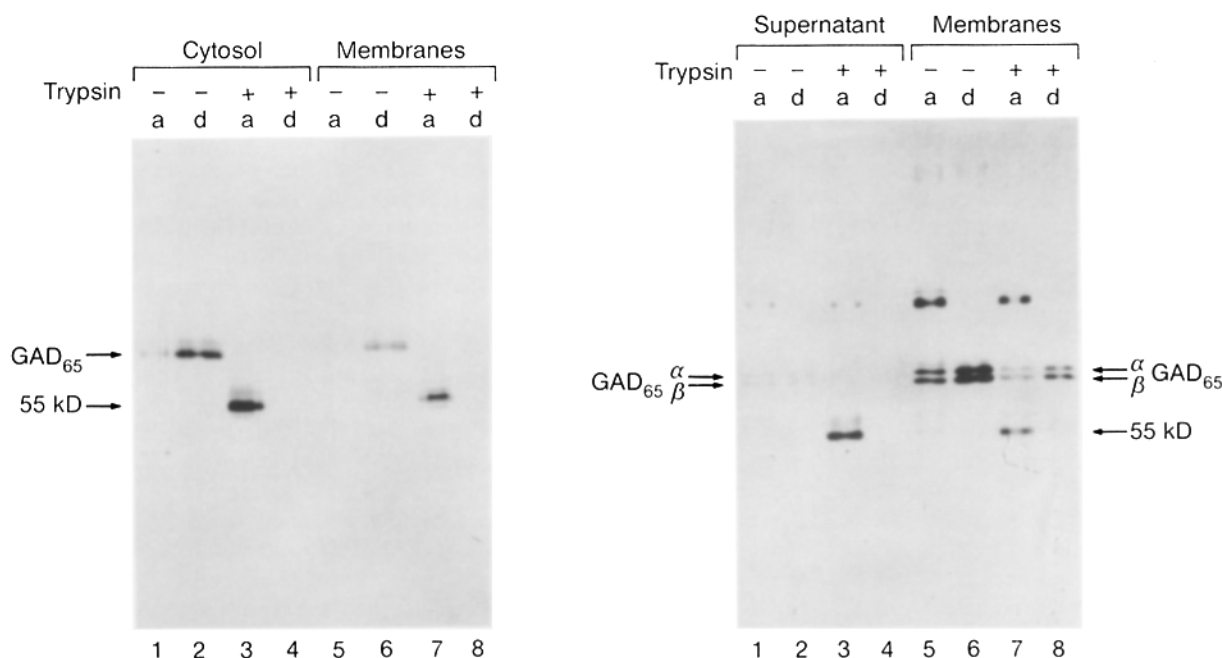
In the initial phases of this study we had assessed whether GAD<sub>65</sub> was membrane anchored by a PIG moiety (for review see Ferguson and Williams, 1988). In this type of membrane anchor, the diacylglycerol moiety of the PIG is inserted into the lipid bilayer of membranes, but can be removed by the action of exogenous or endogenous phosphatidyl inositol specific phospholipase C (PIPLC) (for review see Ferguson and Williams, 1988).

Several lines of evidence, however, led to the conclusion that GAD<sub>65</sub> is not modified by a PIG residue. First, no incorporation of radioactivity into GAD<sub>65</sub> was detected in the presence of <sup>3</sup>H-inositol, <sup>3</sup>H-ethanolamine, or <sup>3</sup>H-glucosamine which are building blocks of the PIG anchor. Second, if the soluble and membrane-bound forms of GAD<sub>65</sub> differed by a PIG moiety, they would be expected to have different isoelectric points, which is not the case (Christgau et al., 1991).

Finally, we analyzed the ability of PIPLC to release GAD<sub>65</sub> from washed islet cell membranes in the presence of buffers with and without components which either inhibit or stimulate the activity of PIPLC. The standard Hepes buffer A, which contains a cocktail of proteolytic and other enzyme inhibitors, was used in control experiments. These experiments revealed that GAD<sub>65</sub> was released from the membranes in all conditions except in buffer A, but equally with and without the addition of PIPLC. Taken together, the results suggest that an endogenous enzyme, which is inhibited in the conditions of Hepes buffer A, can cleave the membrane anchor and release GAD<sub>65</sub>. Exogenously added



**Figure 5.** Time-dependent release of GAD<sub>65</sub> from membranes. Washed membrane fractions prepared from rat islets homogenized in hypotonic conditions were resuspended and incubated in the presence or absence of protease inhibitors for the indicated length of time and then repelleted by ultracentrifugation at 265,000 g. The release of GAD<sub>65</sub> was analyzed by immunoprecipitation of supernatants, SDS-PAGE, and immunoblotting with the 1266 COOH-terminal peptide antibody. GAD<sub>65</sub> released during a 60-min incubation without enzyme inhibitors was subjected to a TX-114 phase separation to analyze the distribution into detergent (d) and aqueous (a) phases (lanes 9 and 10).



**Figure 6.** Absence of hydrophobic and membrane anchoring moieties in a 55-kD-terminal trypsin fragment of GAD<sub>65</sub>. (A) Tx-114 detergent phase was prepared from a cytosol and membrane fraction isolated from rat islets homogenized in hypotonic conditions. The detergent phases were incubated with and without trypsin followed by a new TX-114 phase separation. The detergent phase (d) and aqueous phase (a) from this last separation were analyzed by SDS-PAGE followed by Western blotting and immunostaining with the COOH-terminal peptide antiserum 1266. (B) Washed membranes were prepared from rat islets homogenized in hypotonic conditions resuspended and incubated with and without trypsin followed by ultracentrifugation at 265,000 g. The supernatants and TX-114 extracts of residual membranes were subjected to phase separation and aqueous (a) and detergent (d) phases were analyzed by SDS-PAGE, Western blotting, and immunostaining with the COOH-terminal peptide antiserum 1266.

PIPLC does not, however, affect this release, consistent with the membrane anchor not having the properties of a PIG residue.

To analyze the release of GAD<sub>65</sub> further, washed membranes prepared from islets, were incubated with and without enzyme inhibitors for different periods of time. No release of GAD<sub>65</sub> from membranes was detected in the presence of enzyme inhibitors. In the absence of enzyme inhibitors, however, amphiphilic GAD<sub>65</sub> was released from membranes in a time-dependent fashion (Fig. 5), suggesting that islets contain an enzyme activity which can cleave the GAD<sub>65</sub> membrane anchor, but not the hydrophobic moiety of the soluble S2 form. GAD<sub>65</sub> released from membranes of islet cells did partition into the TX-114 detergent phase (Fig. 5, lanes 9 and 10, see also Fig. 6 B), suggesting that the release represents a conversion of the firmly membrane anchored form to the soluble but still hydrophobic S2 form. In addition a 55-kD hydrophilic fragment of GAD<sub>65</sub> was detected in the fraction released from the membrane in some experiments (Fig. 5, lane 10), suggesting either that the protein can also be released from the membrane by proteolytic cleavage or that released GAD<sub>65</sub> is susceptible to proteolytic cleavage.

#### Localization of the Hydrophobic Membrane Anchoring Region of GAD<sub>65</sub> to a Fragment at the NH<sub>2</sub> Terminal

We addressed the localization of the hydrophobic residue(s) and membrane anchor in the GAD<sub>65</sub> protein by analyzing the hydrophobicity of fragment(s) generated by mild trypsin

digestion of the hydrophobic soluble and membrane forms of GAD<sub>65</sub>. Islets were homogenized in a hypotonic buffer and cytosol and membrane fractions prepared. The amphiphilic proteins in both fractions were purified into the detergent phase of TX-114 and incubated with and without trypsin followed by a new TX-114 phase separation. The detergent and aqueous phases from this last phase separation were analyzed by SDS-PAGE and immunoblotting using an antiserum raised against a 19-amino acid COOH-terminal peptide of the larger brain form of GAD (GAD<sub>67</sub>). Because of an extensive homology at the COOH terminus between GAD<sub>67</sub> and GAD<sub>65</sub> (Erlander et al., 1991), this antiserum recognizes both forms equally well. The results are shown in Fig. 6 A. Trypsin digestion resulted in a 55-kD fragment in both the soluble and the particulate fraction, which was recognized by the COOH-terminal peptide antibody, and therefore contains the COOH-terminal part of the GAD molecule.

In incubations without trypsin, GAD<sub>65</sub> from both soluble and particulate fractions partitioned into the TX-114 detergent phase. The 55-kD fragment from both fractions was, however, exclusively detected in the aqueous phase and had thus lost its hydrophobic moiety (Fig. 6 A). The results suggest that the hydrophobic residue(s) in both the soluble and membrane anchored form reside in a 9–10-kD fragment at the NH<sub>2</sub>-terminus.

We next analyzed whether trypsin was able to release membrane-bound GAD<sub>65</sub>. The particulate fraction was resuspended and washed three times in TBS to remove loosely associated proteins, and then incubated with and

without trypsin for 1 h. Released proteins were analyzed by western blotting (Fig. 6 *B*). Although a minor fraction of full-length amphiphilic GAD<sub>65</sub> is released spontaneously from the membranes in those conditions (Fig. 6 *B*, lanes 1 and 2), the majority of GAD<sub>65</sub> remains membrane-anchored (Figure 6 *B*, lanes 5 and 6). In experiments containing trypsin, GAD<sub>65</sub> was released from the membranes as a 55-kD hydrophilic COOH-terminal fragment (Fig. 6 *B*), indicating that the membrane-anchoring moiety resides in a 9–10-kD fragment at the NH<sub>2</sub>-terminus. Attempts to detect the NH<sub>2</sub>-terminal fragment following trypsin digestion by immunoblotting were unsuccessful. Thus, a small (9–10 kD) fragment was not detected by immunostaining with the polyclonal antibody S3 nor with the monoclonal antibody GAD6. It is conceivable that neither antibody reacts with the NH<sub>2</sub>-terminal fragment or that the fragment is further digested into small fragments by trypsin since it contains several basic residues (Erlander et al., 1991). In attempts to detect the NH<sub>2</sub>-terminal fragment, the trypsin experiments described above and shown in Fig. 6 *A* and *B* were repeated using <sup>3</sup>H-palmitic acid-labeled Sf9 cells and analysis by SDS-PAGE followed by both fluorography of gels as well as immunostaining of Western blots using the COOH-terminal peptide antibody 1266. In those experiments following trypsin treatment, as expected <sup>3</sup>H-palmitic acid labeling was only detected in residual particulate full-length GAD<sub>65</sub> but not in the 55 kD fragment (results not shown). A residual 9–10 kD fragment was detected in fluorograms of total extracts of membranes treated with trypsin but not in membranes from control incubations and may represent the <sup>3</sup>H-palmitoylated NH<sub>2</sub>-terminal fragment of GAD<sub>65</sub> (not shown). Consistently this fragment did not stain with the 1266 antibody on Western blots. In sum the experiments with trypsin provide evidence that the hydrophobic modification and membrane anchoring of GAD<sub>65</sub> takes place in the NH<sub>2</sub>-terminal 9–10-kD part of the protein.

## Discussion

In pancreatic islets GAD<sub>65</sub> undergoes membrane anchoring in a process that involves a two-step modification. The first step results in a hydrophobic molecule which is soluble or has a low membrane avidity. The second step results in a firm membrane anchoring. (Christgau et al., 1991). The results described herein show that the second step involves a hydroxylamine-sensitive palmitoylation, and results in a molecule with intrinsic membrane protein properties. GAD<sub>65</sub> can be released from islet cell membranes in a time-dependent manner, in a process in which enzyme activity is implicated, suggesting that the second step is reversible and that GAD<sub>65</sub> may be flexible in its membrane-anchoring properties. Immunogold EM analysis with GAD antibodies show clear labeling of the membrane of small vesicles in pancreatic  $\beta$ -cells, which are similar in size to synaptic-like microvesicles (Reetz et al., 1991) and clearly distinct from the insulin-containing secretory vesicles in those cells. The EM results shown here are consistent with data from immunohistochemical analysis of GAD and GABA in  $\beta$ -cells and neurons by confocal microscopy (Sorenson et al., 1991; Reetz et al., 1991), which show localization of both to a vesicular compartment. This compartment was shown to be concentrated in nerve terminals and in the distal portions of neurite-like

extensions in  $\beta$ -cells, and was tentatively identified as synaptic vesicles in neurons and synaptic-like microvesicles in  $\beta$ -cells by colocalization with synaptophysin (Reetz et al., 1991). Based on the confocal microscopy data (Sorenson et al., 1991; Reetz et al., 1991), on the electron microscopic analysis shown here, and on the demonstration of the intrinsic membrane protein properties of GAD<sub>65</sub> (Christgau et al., 1991, this study, and unpublished results) we conclude that the membrane-anchored form of GAD<sub>65</sub> is localized to the membrane of synaptic-like microvesicles containing GABA in  $\beta$ -cells and synaptic vesicles in neurons. A previous immunogold EM study using embedded brain and islet-cell homogenates (Reetz et al., 1991) has shown concentrations of GAD immunoreactivity in the proximity of synaptic vesicles in brain and synaptic-like microvesicles containing GABA in pancreatic  $\beta$ -cells. However this study did not reveal an association of GAD with those microvesicles. This previous failure to detect GAD in synaptic vesicle membranes by immunogold EM of homogenized islets, and by subcellular fractionation (Reetz et al., 1991) is most likely because of the reversible nature of the membrane anchoring we have now documented. In fact, preparation of membranes in the absence of enzyme inhibitors and at elevated temperatures or at weak alkaline pH can result in the majority of GAD<sub>65</sub> being recovered as soluble (our unpublished results). Furthermore, even in the absence of trypsin a fraction of GAD is often recovered as a 55-kD soluble and hydrophilic COOH-terminal fragment (Christgau et al., 1991; and this study), suggesting that GAD may also be released from membranes by proteolytic cleavage during homogenization and/or further fractionation of cells.

The characteristics of the posttranslational modifications and the subcellular and amphiphilic properties of the different forms of GAD<sub>65</sub> resemble in many ways those of the ras proteins (Hancock et al., 1989) and the rab GTP-binding proteins (for review see Balch, 1990), all of which exist both in a soluble hydrophobic and a membrane-bound hydrophobic form, with intrinsic membrane protein properties. The latter group includes the rab 3A and 3B proteins which are also found in the membrane of synaptic vesicles (for review see Südhof and Jahn, 1991). The posttranslational modification and membrane anchoring of the ras and the rab proteins, however, involves isoprenylation and carboxymethylation at the COOH terminus which contains either a CAAX, CXC, or CC motif (Hancock et al., 1989; Farnsworth et al., 1991; Kohrsravi-Far et al., 1991). GAD<sub>65</sub> does not contain an isoprenylation motif at the COOH terminus (Erlander et al., 1991) and does not seem to undergo this modification. Rather, the first modification of GAD<sub>65</sub> appears to involve a different mechanism. The second modification of GAD<sub>65</sub>, however, does resemble that of some of the ras proteins in being a palmitoylation that results in firm membrane anchoring. Furthermore, as for ras proteins (Magee et al., 1987), the membrane anchoring is reversible.

Interestingly, GAD<sub>65</sub> can be posttranslationally modified, palmitoylated, and membrane anchored in insect Sf9 cells and in cos cells (our unpublished results), which apparently do not contain synaptic-like microvesicles. This result suggests that the posttranslational modification of GAD<sub>65</sub> and its targeting to synaptic vesicles are separate events. Posttranslational modification and membrane anchoring may take place before budding of membrane vesicles from the

Golgi complex. Then perhaps the final targeting of the membrane-anchored form to synaptic-like microvesicles in  $\beta$ -cells and synaptic vesicles in neurons depends on interaction with components of synaptic vesicles which are absent in Sf9 and cos cells.

### Relevance for Function

In cerebral cortex, both GABA and glutamate have been shown to be stored in synaptic vesicles. It is however unclear whether those amino acids reside in the same or different vesicles (Burger et al., 1991). Membrane-anchored GAD<sub>65</sub> may therefore be localized so as to provide GABA directly to the vesicle that secretes it, thereby coordinating enzymatic activity and GABA secretion. An important question is whether GAD<sub>65</sub> resides in the lumen or at the cytosolic face of the vesicles. In the first case the enzyme would be in the immediate proximity of glutamate and/or GABA. It is notable that GAD<sub>65</sub> lacks a known signal sequence (Erlander et al., 1991) and thus would require a novel transport mechanism to achieve a luminal localization. If GAD is anchored at the cytoplasmic face, which seems most likely, it could associate with its substrate glutamate and release its product GABA in coordination with their transporters in the membrane of synaptic vesicles (Burger et al., 1991 and references therein).

In rat pancreatic  $\beta$ -cells membrane attachment is the exclusive property of the predominant GAD<sub>65</sub> form and is not detected in the minor nonallelic protein, GAD<sub>67</sub>, which remains hydrophilic and soluble throughout its lifespan (Christgau et al., 1991). GAD<sub>65</sub> and GAD<sub>67</sub> are most divergent in the first 100 amino acids but share in high degree of homology in the remainder of the molecules (Erlander et al., 1991). Taking advantage of a trypsin sensitive hot spot, we have now shown evidence that the area of hydrophobic modification and membrane anchoring of GAD<sub>65</sub> resides in the 9-10-kD fragment at the NH<sub>2</sub> terminus where this protein differs most significantly from GAD<sub>67</sub>.

The reason why two genes encoding distinct versions of this enzyme have developed and why  $\beta$ -cells preferably express GAD<sub>65</sub> is not known. GAD<sub>67</sub> binds the coenzyme pyridoxal phosphate more tightly and is less dependent on exogenous coenzyme than GAD<sub>65</sub>, which is mainly found in the apoenzyme form (Martin et al., 1991). Accordingly GAD<sub>65</sub> activity is more sensitive to pyridoxal phosphate levels than GAD<sub>67</sub> (Kaufman et al., 1991). In neurons GAD<sub>65</sub> has been shown to be predominantly localized to nerve endings (Henry and Tappaz 1991; Kaufman et al., 1991) consistent with its localization in the membrane of synaptic vesicles which concentrate proximal to the synaptic membrane. GAD<sub>67</sub> is however predominantly localized in the cell bodies of neurons (Kaufman et al., 1991; Henry and Tappaz, 1991). It is conceivable that the localization of the pyridoxal phosphate inducible form, GAD<sub>65</sub>, to the membrane of the vesicles that store and secrete its product GABA has a regulatory purpose and serves to accommodate sudden increases in GABA demand. Interestingly there are examples of other enzymes involved in the synthesis of transmitters that are anchored to the membrane of granules that store and secrete them. Thus tyrosine hydroxylase, the initial and rate-limiting enzyme in the biosynthesis of the catecholamines, and dopamine  $\beta$ -hydroxylase, the final enzyme in the synthesis of norepinephrine, exist in both soluble and membrane-

bound forms and the latter have been localized to the membrane of chromaffin granules in the adrenal medulla (Kuhn et al., 1990; Bon et al., 1991).

### Relevance for Autoimmunity

The correlation in autoimmunity toward pancreatic  $\beta$ -cells and brain neurons is remarkable, especially as it is revealed in the development of autoantibodies which recognize the same antigen, the 64-kD form of glutamic acid decarboxylase. A second nonallelic form of this enzyme encoded by a distinct gene, GAD<sub>67</sub>, is apparently only of secondary importance. GAD<sub>67</sub> lacks the primary epitopes which are antigenic in SMS (Baekkeskov et al., 1990), and evidences a lower incidence of specific autoantibodies in IDDM (our unpublished results). It is therefore provocative that these two proteins are clearly distinguished by an NH<sub>2</sub>-terminal region which determines hydrophobic modification, subsequent palmitoylation, and consequent membrane anchoring and subcellular localization. Only GAD<sub>65</sub> has these two posttranslational modifications that confer hydrophobicity, membrane attachment, and association with microvesicles. It is of note that palmitoylated influenza peptides induce potent cytotoxic T-cell responses in vivo whereas their nonpalmitoylated counterparts do not (Deres et al., 1989). Perhaps one of the posttranslational modifications, or the selective localization of GAD<sub>65</sub> into a membrane compartment that is secretory in nature is instrumental for its role as an autoantigen in these two diverse but coinciding autoimmune diseases.

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